Honors Research Distinction Thesis

Using Optical Mapping to Study the Role of CaMKII in Ischemia/Reperfusion Injury

Submitted to:

The Engineering Honors Committee

College of Engineering

244 Hitchcock Hall

2070 Neil Avenue

Columbus, Ohio 43210

Prepared by: Tony Satroplus

Project Advisor: Dr. Thomas Hund

Committee Members:

Thomas Hund, Ph.D., Assistant Professor Biomedical Engineering

Rengasayee Veeraraghavan, Ph.D., Professor Biomedical Engineering

Submission: April 20th, 2017

Abstract

Heart disease is a major cause of death in the United States and a majority of the deaths are due to sudden cardiac death (SCD). A subset of these deaths are due to ventricular fibrillation, a type of cardiac arrhythmia that is often a consequence of cardiac ischemia and reperfusion. Understanding the mechanisms behind cardiac arrhythmias will improve the knowledge available for the development of clinical treatments. This study aims to focus on the molecular mechanisms underlying arrhythmia in the setting of ischemia and reperfusion (I/R). Specifically, my works test the hypothesis that I/R induced arrhythmias depend on the action of the multifunctional Ca^{2+}/calmodulin dependent kinase II (CaMKII) on the voltage-gated sodium channel $Na_v1.5$. Previous studies have identified a specific amino acid (Ser571) on $Na_v1.5$ that is targeted by CaMKII, with implications for channel gating and cell excitability. The role CaMKII plays in arrhythmias will be studied through novel mouse models with genetic manipulation of the Ser571 site in $Na_v1.5$ (S571A and S571E mice), together with wildtype mice as a control. Optical mapping, a modern approach to studying the electrophysiology of the heart in real-time, will be used with an ischemia and reperfusion protocol. The studies revealed a prolongation of action potential duration (APD), increased recovery time, and increased arrhythmia susceptibility in S571E and WT mice versus S571A mice. Selectively targeting this CaMKII-dependent pathway to decrease late sodium current may have clinical application for reducing arrhythmias in acute ischemia and reperfusion settings.

Acknowledgements

I would first like to thank my thesis advisor Dr. Thomas Hund of the Department of Biomedical Engineering at The Ohio State University. The door to Dr. Hund's office was always open and he was supportive during my four years as an undergraduate in his lab. He consistently allowed this paper to be my own work, but steered my in the right direction whenever I needed it. During my defense, many great questions were brought up to further my studies in the field.

I would also like to thank Dr. Rengasayee Veeraraghavan of the Department of Biomedical Engineering at The Ohio State University as the second reader of this thesis. He brought up great questions and suggestions during the revisions of the paper and proposed challenging questions during the defense to improve my understanding of my work and the field of excitable cells in general.

Contents

List of Figures

1. Background and Motivation

Heart Function

A coordinated contraction of the heart must occur for blood to reach the rest of the body. The electrophysiology of the heart is responsible for propagating an electrical signal throughout the organ to produce the coordinated contraction. Cardiomyocytes rest at a membrane potential of -85 mV with large extracellular sodium concentrations and high intracellular potassium concentrations.[1] When a stimulus is received, voltage gated sodium channels allow the sodium ions into the cell and increase the potential of the inside of the cell, creating a depolarization. During depolarization, the L-type calcium channels release calcium into the cytoplasm from the sarcoplasmic reticulum, creating calcium sparks in the cell.[2] During repolarization, most voltage gated sodium channels are inactivated, meaning no matter the potential of the cell, they will not open. During this time potassium currents and currents produced by the sodium calcium exchanger (NCX) start repolarization. Depolarization is initiated at the sinoatrial node and propagates into the atria and ventricles through gap junctions.[2] The propagation of the signal initiates a coordinated contraction to pump blood to the body.

Myocardial Infarction

In the United States, 1 million people suffer from myocardial infarction annually, including the 700,000 patients who are treated with a cardiac procedure and fall into cardioplegic arrest.[3] In recent years, myocardial infarctions have become significant causes of death in developing countries as well.[4] A myocardial infarction, commonly known as a heart attack,

occurs when a blood vessel in the heart, usually an artery on the epicardium, becomes blocked by an upstream plaque fragmenting from the vessel wall.[4] In the clinical setting, diagnosis of a myocardial infarction comes from increased troponin levels in the bloodstream, an indicator or cardiomyocyte necrosis due to ischemia and induced hypoxia.

During a myocardial infarction, the rapid restoration of normal coronary circulation is critical in reducing permanent damage to the organ caused by hypoxia. The combination of acute treatments involving pharmaceutical remedies and mechanical treatments, including using catheters to break up the blockages, and long-term treatments involving antithrombotic medication and lifestyle changes aim to prevent fatalities from the disease.[4] However, the actual process of reperfusing the heart has been shown to cause physical damage, harm from oxygen radicals, an influx of calcium into the cells, and inflammation, all leading to electrophysiological abnormalities such as arrhythmia, and more specifically, ventricular tachycardia.[5] Other deleterious effects of the cardiac procedure are the increasing rate of necrosis, swelling of the cells, and a further decrease in effective function of the ventricles.[6] While the ischemia itself can lead to long-term disease of the heart such as necrosis and heart failure, the rapid reperfusion following an acute myocardial infarction has been shown to lead to fatal ventricular arrhythmias, termed “reperfusion arrhythmias”.[7]

Potential Mechanisms involved in Ischemia/Reperfusion Injury

Previous research on identifying potential contributors to ischemia-reperfusion injury have come up with plausible mechanisms.[8] The Na^+/Ca^{2+} exchanger (NCX) is responsible for the effusion of calcium across the plasma membrane without the consumption of energy.[9] The forward mode for NCX extrudes one calcium ion in exchange for the influx of 3 sodium ions, while the reverse mode does the opposite, actually bringing calcium into the cell – bi-directionality of the exchanger has been shown in previous studies.[10] The link between calcium influx and I/R injury has been shown, but the mechanism has yet to be elucidated.[11] At one point, protein kinase A was considered to be the upstream cause for phosphorylation of the NCX, increasing the exchange activity, but recent evidence suggests no specific phosphorylation site has been confirmed, indicating PKA is not directly responsible for the activity of NCX and its possible role in IR injury.[12]

A significant step leading to increased reverse mode NCX function is intracellular sodium overload.[13] One mechanism behind the influx of sodium is that it is the result of the cells performing cellular respiration in a hypoxic environment. While normal cellular respiration by cardiomyocytes is aerobic, relying on a readily available supply of oxygen, during an acute myocardial infarction, the tissue is not supplied with oxygen. The cells are forces to turn to anaerobic cellular respiration, inducing acidosis within the hypoxic region of the tissue. The increase in H^+ concentration activates the sodium hydrogen exchanger 1, the isoform of the exchanger most commonly found in cardiomyocytes.[14,15]

Voltage-gated sodium channels (Na_v) play a major role in the rapid depolarization of the cardiomyocytes due to a quick influx of sodium ions into the cell.[16] The rapid depolarization of the cardiomyocytes is essential for coordinated contraction of the organ and efficiency of the

organ to pump blood throughout the body. When Na_v are activated under normal conditions, a quick influx of sodium quickly depolarizes the cell. This Na^+ current (I_{Na}) precedes the inactivation of most Na_v in healthy tissue, preventing the channels from opening for a certain period of time independent from membrane potential and allowing for repolarization in preparation for a future contraction.[17] During normal function, a small percentage of cardiac Na_v channels fail to inactivate, causing a late sodium current to persist ($I_{Na,L}$) throughout the AP. While in the non-disease states, this $I_{Na,L}$ does not interrupt regular heart rhythm, a high $I_{Na,L}$ can increase action potential duration and the ability for the heart to enter into arrhythmia.[18] On the cellular level, ischemia has been shown to increase resting membrane potential and, initially, increase the action potential duration – all of which can lead to arrhythmia on an organ level.[19] Both cellular and organ level experiments have supported the notion that the accumulation of sodium post-IR is due in part to an increase in late sodium current.[19–25] The accumulation of sodium in the cytosol after ischemia was shown to be reduced by blocking sodium currents on a cellular level using RAN and TTX.[19]

Role of CaMKII-dependent phosphorylation of Nav1.5 at Ser571 site

Many isoforms of Na_v are found throughout the body. While this study focusses on $Na_v1.5$, other isoforms have been notes to contribute significantly to I_{Na} and $I_{Na,L}$, including neuronal and skeletal muscle isoforms.[26] It should be noted that the findings from this study should be put into perspective as a small piece of the complex cellular system that leads to arrhythmia.

Previous studies have identified the multifunctional serine/threonine kinase CaMKII to have diverse functionality in the heart.[27] Transcription factors, voltage-gated ion channels, and transporter proteins are some of the few cellular components affected by CaMKII.[28] Previous research has suggested that a strong connection is present between CaMKII expression and cardiac pathology,[28] specifically, CaMKII expression levels and/or activity have been shown to increase in heart failure patients.[29] Previous work in our lab has shown CaMKII phosphorylates the Ser571 site on $Na_v1.5$ to increase $I_{Na,L}$.[30–33] With $Na_v1.5$ being a major sodium channel involved in producing the cardiac action potential, its modification due to CaMKII can have significant physiological implications including arrhythmia, heart failure, and other causes of sudden cardiac death.[33–35]

Specific studies of $Na_v1.5$ activity have focused on the regulation of its activity affecting late sodium current ($I_{Na,L}$).[33,36,37] The increase in $I_{Na,L}$ has been linked with prolonged action potentials and abnormal ion regulation, leading to events of ectopy at the cellular and organ level.

The understanding about the mechanisms involved in the role CaMKII is not yet clear. Elucidating these mechanisms could reveal opportunity for reducing the possibility of injury caused by ischemia/reperfusion, paving the way for treatment of pathologies that cause such ischemia in the future.

2. Significance

This study has further elucidated on the mechanism of IR injury.To study this mechanism, optical mapping was used mapping as a novel approach to better understanding the

mechanism involved in the effect the expression/inhibition of CaMKII has on ischemic/reperfusion injury. Specifically, CaMKII-dependent phosphorylation of $Na_v1.5$ at the Ser571 site is shown to prolong action potential duration (APD) and increase arrhythmia inducibility following ischemia/reperfusion in knock-in hearts from different mouse models. Obstructing the CaMKII-dependent phosphorylation of the Ser571 site on $Na_v1.5$ by removing the phosphorylation site or blocking the site with mexiletine, inhibiting late Na^+ current) counteracted the increased APD and decreased arrhythmogenesis in excised hearts when exposed to ischemia and reperfusion. A better understanding of the effects and mechanism in this relationship will add to our knowledge on cardiac arrhythmia.

3. Research Goals

Two mouse models were previously developed to study the *in vivo* role of the CaMKII on the regulation of the voltage gated sodium channel $Na_v1.5$: 1) the S571E mouse model inserts a an amino acid that mimics a permanently phosphorylation site in place of the serine at position 571; and 2) the inability of the 571 position site to be phosphorylated is accomplished by replacing the serine cite with an alanine producing the S571A mouse model.[36] The position 571 mentioned above is an amino acid position on the sequence coding for the $Na_v1.5$, which has been shown to be a key site for phosphorylation and regulation of the sodium channel.[38]

Using the previously developed mouse model, this study looks to test the roles CaMKII plays in cardiac arrhythmias induced by ischemia/reperfusion. By comparing the measurements from the S571E to those from the S571A mice, we expect to develop a greater understanding of the role CaMKII plays in cardiac arrhythmias. Based on previous studies of the S571E mouse model, we predict this phenotype will be more vulnerable to injury from ischemia/reperfusion

because of the phosphorylation of the Ser571 position in the $Na_v1.5$ sequence. However, the lack of ability for this same site to be phosphorylated in the S571A mice is predicted to better protect these mice from injury due to ischemia/reperfusion.

4. Methodology

Mouse Models – The following mouse models were used: *Scn5a* knock-in mice (C57/Bl6 background) with S571E or S571A point mutation in $Na_v1.5$ or wildtype (WT) controls [36]. Adult male mice were anesthetized by isoflurane, confirmed by the absence of response to probing of the lower extremities. The mice were subcutaneously injected with Heparin to avoid blood clots during extraction and cannulation. The whole heart was extracted and perfused via the aorta, using a rolling pump at 2 mL/hr. The oxygenated Tyrode's solution, maintained at 36.5°C and titrated to a pH of 7.4 using 5 M hydrochloric acid, contained 140 mM NaCl, 1.0 mM $MgCl_2*6H_2O$, 1.2 mM NaH_2PO_4, 4.0 mM KCl, 1.8 mM $CaCl_2*2H_2O$, 5.6 mM glucose, and 10 mM HEPES. Studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health following protocols that were reviewed and approved by the Institutional Animal Care and Use Committee at The Ohio State University.

Optical Mapping of ex vivo heart preparations – Optical mapping techniques were used to measure the voltage on the anterior portion of the isolated heart. The heart was perfused for 15 minutes with Blebbistatin (from a stock of 5mg/mL in DMSO) to decrease motion for clearer signal production, without significantly altering the electrophysiology [39]. Perfusion continued with the voltage dye di-4-ANEPPS (4 uM) for 15 minutes [40,41]. Images were taken to ensure the

dye reached the extremity tissues of the organ. The fast voltage-sensitive dye was chosen based on previous work showing good signal to noise ratio, negligible light interference, and the reduction of autofluorescence signals that could create inaccurate readings [42]. Global ischemia was induced by stopping perfusion for 15 minutes followed by reperfusion with the Tyrode/Blebbistatin solution for the remainder of the experiment.

To excite the dye, a laser (LEX2-LZ4-G (SciMedia)) was used in conjunction with a 531/40 filter. Signals were collected at 1000 Hz (1ms/frame) using MiCAM05 CMOS cameras and filtered with a 600 nm longpass filter. Resulting images were 1.06x1.06 cm^2 in area with 100x100 pixels. A custom MATLAB code (provided by Dr. Steven Poelzing) was used analyze the signals measured during experimentation. Pacing was performed using a unipolar lead at the center of the anterior heart wall. The pacing protocol used paced the excised heart at 150 ms cycle lengths for 2 seconds (S1), with an extra cycle following with a shorter cycle length (S2). S1S2 protocol was used to induce arrhythmia whereby the S1S2 interval was decreased by 10 ms per measurement until 1:1 capture was not possible or ventricular tachycardia was observed. For this study, ventricular tachycardia was defined as three or more spontaneous beats in rapid succession (less than 100 ms apart).

Immunoblotting – Ventricular lysates were analyzed by SDS-PAGE and immunoblot, as described [32,36,43,44]. Equal protein loading was achieved using standard BCA protocols and verified by Ponceau staining of immunoblots. The following antibodies were used: Total CaMKII (Badrilla), Phospho-CaMKIIδ antibody (Santa Cruz), total and phospho-$Na_v1.5$ (Ser571) [32].

Data and Statistical Analysis –Sigmaplot 12.0 was used for statistical analysis. One-way ANOVA was used for multiple comparisons with the Tukey test for post hoc testing (data presented as mean±SEM). Contingency data were analyzed using a Chi-Square test. The null hypothesis was rejected for $P<0.05$.

5. Results

Ischemia/Reperfusion leads to CaMKII phosphorylation of $Na_v1.5$ and generation of arrhythmias

The central hypothesis of my study is that an increase in CaMKII-dependent phosphorylation of $Na_v1.5$ increases $I_{Na,L}$ and arrhythmias during the ischemia/reperfusion protocol (I/R) (Figure 1). To test the hypothesis, excised hearts from WT, S571A, and S571E mouse models were perfused using a Langendorff system and the entire organ was exposed to ischemia and reperfusion. The baseline effects of I/R were determined by comparing the abundance of total and phosphorylated CaMKII and $Na_v1.5$ in the WT hearts before ischemia, post-ischemia, and post-reperfusion. Previous studies have shown an increase in phosphorylated-CaMKII during early stages of reperfusion post-ischemia.[30] The studies in this thesis confirm the previous findings of elevated p-CaMKII post-ischemia when compared to baseline, and a normalization of levels post-reperfusion shown in Figure 2A.

Previous studies have shown that CaMKII phosphorylates $Na_v1.5$ at the serine 571 site and that CaMKII activity increases in response to ischemia.[28,33,43,45] Based on these data, I hypothesized that global ischemia would promote increased levels of phosphorylated $Na_v1.5$ with normalization of the levels post-reperfusion. Consistent with this hypothesis, global ischemia was found to increase levels of p-CaMKII and p-$Na_v1.5$, with both returning to normal when the heart is reperfused (Figure 2).

Phosphorylation at S571 prolongs APD and leads to higher arrhythmia susceptibility following I/R

Knock-in mouse models with phenotypes shown in previous studies were used to further clarify the connection between the CaMKII-dependent phosphorylation of $Na_v1.5$ and IR injury induced arrhythmias.[43] A wild type mouse was used as a control with the Serine 571 site unchanged. Two modified mouse models were used: 1. The serine at the phosphorylation site of Nav1.5 (S571) was replaced with a glutamic, creating a phosphomimic model (S571E). 2. The S571 was replaced with an alanine to prevent any phosphorylation at the specific site (S571A). The action potentials of five excised hearts were measured for each mouse model. Typical cardiac action potentials show a higher average potential during repolarization[17,46], while studies action potential measurements of the entire organ align with the sharper return to a polarized state earlier in the recovery period.[41,47–49] Measuring action potential duration (APD) through optical mapping revealed a significantly longer action potential in the S571E mouse model during baseline, post-ischemia, and post-reperfusion compared to that of the WT and S571A. A significant difference in APD of the baseline and S571A was seen immediately following ischemia, showing a significantly shorter APD in the S571A. While not always statistically significant, the data illustrates a trend of the S571A having a shorter APD compared to the WT Figure 3B. The overall reduction in APD due to IR is consistent with studies measuring ATP sensitive potassium channels, assuming a lower concentration of ATP in the tissue because of the tissue relying on anaerobic respiration.[49]

Following the APD measurements, the generation of arrhythmia by IR was measured for WT, S571E, and S571A excised hearts using an S1S2 protocol. The measurements of the WT illustrated in Figure 4A is an arrhythmia occurring after stimulation by the shorter S2 wave (location shown with the arrow). An ideal heart, as shown in the S571A illustration, would

contract during the S2 wave, but return to resting potential after the contraction. Arrhythmia inducibility in the WT and S571E mice was high, with 80% and 100% rates of incidence (Figure 4B). Statistically significant differences between the incidences of WT and S571E compared to incidences of the S571A were found (p-value<0.05, $n=5$). The time between the final S1 and the S2 stimulations being shortened until the heart did not contract for the S2 pulse. While the S571E and WT mice went into arrhythmia long before the beat was lost, the S571A continued to contract and quickly return to resting potential for the increasingly short temporal distances between the S1 and S2 pulses, showing protect against potential arrhythmia inducing abnormalities. The models revealed that not only does phosphorylation at the $Na_v1.5$ S571 increase susceptibility to arrhythmia, but blocking this phosphorylation actually protects the heart from such electrophysiological abnormalities. The data supports the hypothesis that CaMKII-dependent phosphorylation of the S571 site in $Na_v1.5$ alters excitability and promotes arrhythmias following ischemia and reperfusion.

Treatment with Mexiletine shortens APD and reduces arrhythmia susceptibility following Ischemia/Reperfusion

Previous studies have shown the ability of Mexiletine to inhibit $I_{Na,L.}$ WT hearts were treated with Mexiletine, a selective Na^+-channel blocker, during the IR protocol to determine if late sodium current inhibition could reduce the number of arrhythmic events. While similar studies have been done with ranolazine, the mexiletine was used to target a more specific pathway of arrhythmia induction. The measurements of the change in AP compared to baseline would test the hypothesis that following I/R, CaMKII-dependent phosphorylation of the $Na_v1.5$ S571 site provides substrate for arrhythmia through increased $I_{Na,L}$ (Figure 5). A Mexiletine

concentration of 10 μM was used because if the evidence supporting the block in $I_{Na,L}$ without significant disruption of the peak sodium current.[37] While the concentration used was taken from previous studies in single cells, this may not translate to the whole-organ level. Representative traces of the action potentials of each group of excised hearts is shown in Figure 5A. Mexiletine shortened APD and promoted faster recovery during reperfusion compared to the untreated group. The WT hearts treated with the drug mimicked the APD patterns of S571A mice, including a significantly reduced APD post-ischemia and a rapid recovery of the APD post-reperfusion, suggesting protection from IR injury via inhibition of the $I_{Na,L}$ in the baseline mice.

Shown in Figure 6A is the optical mapping traces for both the baseline hearts treated with Mexiletine and those as the control for the experiment. The WT without treatment is a representation of ventricular tachycardia (VT) in the mouse model. For the studies in this paper, VT was ventricular arrhythmia sustained for longer than 4 additional depolarizations of the ventricle, while altered ventricular excitability was arrhythmia-like contractions that subsided at or before three peaks after the S2 pulse had been delivered. The VT inducibility was significantly (n=5) higher in the WT mice without treatment (Figure 6B), with VT patters in the non-treated WT being consistent with previous optical mapping research involving VT.[50] In the context of this study, Mexiletine is shown to reduce arrhythmogenicity in the excised baseline hearts.

The results above are consistent with the hypothesis that CaMKII-dependent phosphorylation (activation) of the $Na_v1.5$ S571 site increased $I_{Na,L}$ to foster deleterious excitability and arrhythmias during ischemia and reperfusion.

6. Discussion

Reperfusion of the coronary arteries and surrounding vessels in the heart following a myocardial infarction (MI) is necessary to limit necrosis and other toxic effects of hypoxia in the cardiac tissue.[5,51,52] Still, with approximately one million patients suffering from myocardial infarction annually in the US,[4,5,53,54] the mechanism behind the more likely development of arrhythmias due to IR injury is not completely understood.[5,8,55,56] Many groups have attempted to limit intracellular Ca^{2+} overload due to concentrations of Na^{+} above physiological levels, but identifying important steps in the pathway without altering normal ion currents, which would cause deleterious side effects, has proven to be difficult. Late sodium current ($I_{Na,L}$) has been suggested as a mechanism in calcium overload, but clear evidence supporting the connecting mechanism has not been provides. This study identifies the mechanism upstream of $Na_v1.5$ that connects the IR injury with the downstream effects on arrhythmia susceptibility through accumulation of intracellular Na^{+}. Immunoblotting revealed an increase in phosphorylated $Na_v1.5$ following ischemia together with increased phosphorylated CaMKII.

Using single cell patch clamping experiments and other cell-based experiments, studies have shown there to be a connection between CaMKII and $Na_v1.5$, with the consensus being CaMKII phosphorylates the S571 site on $Na_v1.5$ to increase activation of the channel.[27,33,36,43,57] However, this study shows coinciding increases in p-CaMKII and p-$Na_v1.5$ at the S571 site during IR injury. The results support previous findings in cell-based experiments, shrinking the gap in knowledge between such experiments and whole-organ experiments as performed here. The studies were further supported by the knock-in mouse models (S571E and S571A). The increase in $I_{Na,L}$, arrhythmogenicity of the tissue, and cell excitability show in the S571E support the hypothesis that CaMKII-dependent regulation of the above characteristics of the heart,

necessitates the use of the Ser571 site on $Na_v1.5$. The results indicate the prevention of CaMKII-dependent phosphorylation of $Na_v1.5$ at the Ser571 site increases the speed and ability of the heart post-ischemia, and protects the organ from arrhythmia during IR.

The study of the effect of Mexiletine during IR revealed protection against arrhythmia during the protocol, indicating the therapeutic effect of inhibiting the phosphorylation of the Ser571 site and activation of $Na_v1.5$. Both lidocaine and mexiletine have proven to be efficient in the treatment of ventricular tachycardia, with mexiletine being the orally taken medication.[17,18,27,58–60] While IR injury can persist for longer periods of time than these experiments, limiting IR injury during reperfusion for acute myocardial infarction could be accomplished with small doses of sodium channel blockers such as mexiletine, restricting usage to short periods of time to avoid long-term side effects.[61]

The data from the study is consistent with the hypothesis that precursors to arrhythmia are enhanced during the CaMKII-dependent hyperphosphorylation of $Na_v1.5$ at the S571 site during IR. The pathway the increased arrhythmias due to hyperphosphorylation of $Na_v1.5$ is surmised to be an increase in $I_{Na,L}$, leading to sodium accumulation, and intracellular Ca^{2+} overload.

CaMKII has been shown to contribute to the production of cardiac arrhythmia in heart failure and chronic disease states.[31,62–65] While increased CaMKII activity is shown to incite abnormal cardiac excitability and function, these studies support many causes of the increased activity including β-adrenergic stimulation, oxygen radicals, and the production of inflammation. The role of CaMKII in chronic disease states has been widely investigated.[66] Additionally, further understanding the role of CaMKII plays in acute disease states such as IR injury and acute myocardial infarction could elucidate further clinical treatments for symptoms like

arrhythmia and VT.[30,46,47,56,67] To investigate the role acute CaMKII activation plays in IR injury, studies have showed increased levels of phosphorylated CaMKII with significant effects on downstream targets PLN and RyR2 in experiments lasting as little as 15 minutes.[30,47,68] The studies above mentioned increased phosphorylation of PLN and RyR2 following IR, with downstream impact on Ca^{2+} influx and efflux through the sarcoplasmic reticulum. Both these effects, and the Na^{+}/Ca^{2+} overload due to $I_{Na,L}$ contribute to the accumulation of Ca^{2+} in the cardiomyocyte cytoplasm and arrhythmogenesis in the heart post-IR.[11,67] The experiments in this study are consistent with the literature, showing similar increases in p-CaMKII following the 15-minute period of ischemia in parallel with arrhythmogenesis of the excised hearts. While previous studies have focused on the direct connection of CaMKII to the intracellular Ca^{2+} overload following IR, these novel studies integrate CaMKII-dependent phosphorylation $Na_v1.5$ as a mechanism of increasing Na^{+} accumulation leading to Ca^{2+} overload.

The acute stress on the excised hearts in the experiments reveal upregulation of phosphorylated $Na_v1.5$ after only 15 minutes, with normalization of regulation during the 15-minute reperfusion cycle. Other work is consistent with these results, illustrating Na^{+} overload in rat hearts after only 5 minutes of ischemia, with more drastic levels as the period of ischemia is lengthened up to 30 minutes.[69] While the recovery of proper Na^{+} was slow following longer ischemia events, intracellular concentrations of Na^{+} were shown to normalize in as little as five minutes.[69] With the shown induction of acidosis during ischemia, the Na^{+}/Ca^{2+} exchanger (NCX) could cause the overload of intracellular Na^{+}.[70] However, another likely contributing cause is the increase in $I_{Na,L}$ during IR.[19,23,36,71] In this study, the upregulation of p-$Na_v1.5$ is shown during the 15-minute ischemia protocol. However, exposing the heart to ischemia for shorter and incremental time periods, could further reveal the role of late sodium current in the mechanisms

of Na^+ overload. Additionally, the variation in time ischemia and reperfusion would reveal important changes in CaMKII activity along with other Ca^{2+} handling proteins.

It is important to note that $I_{Na,L}$ must be protective in some instances. The upregulation of the $I_{Na,L}$ after acute ischemic events, has been shown to reduce reentry into arrhythmia because of APD prolongation, counteracting the shortening of the APD shown during IR, and to allow Ca^{2+} into the cell to increase systolic efficiency of the heart.[72] This connection between APD prolongation and increase in $I_{Na,L}$ is supported by the results of this study (Figure 3). The APD of S571A hearts post-ischemia are decreased more drastically than that of the WT or S571E mice. Since the phosphorylation site for Nav1.5 is ablated in the S571A mice, the significant decrease in APD can be attributed to the inability for $I_{Na,L}$ to counteract this effect. With temporal changes in the exposure to ischemia, a tipping point for the deleterious effects of $I_{Na,L}$ to become significant could be revealed. Acute myocardial infarctions could be dissected into degree of acuteness.

The investigation of the heterogeneity of the heart should also be conducted. The expression of $Na_v1.5$ in just cardiomyocytes is shown to be nonuniform across the epicardium and endocardium of the mouse ventricle.[35,73] Differences in $Na_v1.5$ expression and therefore, amounts of $I_{Na,L}$, would affect the origin or path of cardiac ectopy and possibly creating nonuniform arrhythmogenicity across regions of ventricular tissue. Higher concentrations of $Na_v1.5$ found in the endocardium increase the ability for this region to be vulnerable to Na^+ and Ca^{2+} overload following IR. On a similar note, with different regulation of $Na_v1.5$ throughout the heart, the dispersion of action potentials during IR could be affected. Conduction blocking regions could form, causing increased APD throughout the heart. The study of $NA_v1.5$ distribution in the heart following IR will give more insight into this investigation.

Many studies have been conducted with the goal of inhibiting the reversal of the NCX in cardiomyocytes to prevent the Ca^{2+} overload response form increasing levels of intracellular Na^{+}, with the main goal of the heart being less susceptible to arrhythmias.[11,74,75] However, the intracellular Na^{+} overload upstream of the NCX due to $I_{Na,L}$ during IR injury has not been given as much attention. While, the increase in activity of $I_{Na,L}$ has been shown to promote intracellular Na^{+} overload, the observance of this phenomenon in IR injury has not been fully explored.[18,19,72,76,77] This phenomenon could connect the arrhythmogenesis present in IR injury with abnormal activity of $I_{Na,L}$. Furthermore, experiments have revealed the activity of $Na_v1.5$ is dependent on CaMKII phosphorylation, with higher activity of CaMKII showing parallel increases in $I_{Na,L}$.[23] Although previous studies have shown the connection between CaMKII and activation of $NA_v1.5$, the experiments here go further in successfully identifying CaMKII-dependent phosphorylation of $NA_v1.5$ at the Ser571 site upstream of increased $I_{Na,L}$ and Na^{+} overload in IR injury. This specification in phosphorylation site was studied through the use of novel S571A and S571E knock-in mice, making possible the complete phosphorylation or ablation of the Ser571 phosphorylation site, showing changes to $I_{Na,L}$ and $Na_v1.5$ activity. Temporal changes in repolarization of the heart and changes in inducibility of arrhythmias were present during IR with the Ser571 modifications. The data in the study coincide with previous research and support the notion that CaMKII-dependent phosphorylation of $Na_v1.5$ plays an integral role in the upstream mechanism which leads to arrhythmia throughout IR injury.

7. Conclusion

Clinical Implications

Progress has been made in the treatment of myocardial infarctions with the focus on limiting necrosis and IR injury.[4,5,77] Still, a great deal of improvement is needed to prevent reentry arrhythmia and heart failure caused by IR injury.[78] Important attempts at improving recovery from acute myocardial infarction include using drugs such as flecainide and encainide to block Na_v channels. These treatments showed a reduction in the number of premature ventricular contractions, a phenomenon known to trigger ventricular tachycardia,[79] but long-term use was associated with higher inducibility of fatal reentrant arrhythmia.[80] Devices such as pacemakers and implantable AEDs can be helpful to patients recovering from myocardial infarctions, but the challenges of the devices such as cost, durability, and invasiveness reduce the practicality of their use.[81] Since inflammation has been present in IR injury studies, non-specific anti-inflammatory steroids have been used as potential treatment to prevent IR injury-based arrhythmias, but the studies revealed deleterious effects of ventricular function and clinical outcomes by the drugs. [6,51] Some treatments have focused on blocking certain Na_v channels, such as using Ranolazine, to inhibit reentry arrhythmia as a symptom of IR injury..[77,82–84] However, isolating the mechanisms of the drug's ability to inhibit reentry arrhythmia is important to developing a "cleaner" drug with a more specific target. By understanding the specific mechanisms responsible for IR injury following a myocardial infarction could give insight into more specific treatments.[52] This study shines light on the necessity for exploration of the pathogenic pathway involving CaMKII-dependent phosphorylation of $Na_v1.5$ as a result of IR injury. The results suggest the use of drugs like Mexiletine in low doses to target $I_{Na,L}$ during

acute instances of ischemia and reperfusion. Only using the drugs for short term treatments should limit the side effect of inducing reentrant arrhythmias.

Limitations

Limitations in this study provide future directions in the study of the link between CaMKII-dependent phosphorylation of $NA_v1.5$ and IR injury. Most importantly, the IR protocol used in the study created global ischemia. In episodes of natural myocardial infarction, a specific region of the heart is subjected to ischemic conditions, creating gradients of hypoxia throughout the organ. Still, there is no reason to predict that the behavior of the heterogeneous ischemic conditions should alter the CaMKII mechanisms shown in the globally ischemic study. Another obvious limitation is the differences in electrophysiology between the mouse model used and a that of a human.[59] However, studies support the notion that the CaMKII pathway studies here is applicable across species.[18,20]

Future Studies

Further experimentation is necessary to completely understand the pathway leading to IR injury via Ca^{2+}/Na^{+} overload. By changing the time of the ischemia/reperfusion protocol, the upregulation of p-CaMKII and p-$Na_v1.5$ can be better understood. Studying levels of these phosphorylated proteins as well as time taken for levels to normalize post-ischemia could give insight into when IR injury is most harmful. Additionally, the source of Ca^{2+} to create the Ca^{2+} overload is not completely understood. Initially, a calcium dependent dye will be used to confirm the influx of Ca^{2+} during IR. Three possible sources of the Ca^{2+} overload are L-type calcium

channels, the reverse function of the sodium calcium exchanger (NCX), and release of Ca^{2+} by the sarcoplasmic reticulum.[11,41,85] In future experiments, I will be blocking the NCX, using blockers with previously established efficacies, to study if significant differences in results are observed, suggesting the degree of significance the NCX has in the mechanism leading to Ca^{2+} overload.[86,87] Finally, temporarily blocking of the coronary artery in the mouse model could show more the realistic ischemic heterogeneity throughout the organ.[88]

References:

1. Weidmann, S. Heart: electrophysiology. *Annu. Rev. Physiol.* **36,** 155–169 (1974).
2. Santana, L. F., Cheng, E. P. & Lederer, W. J. How does the shape of the cardiac action potential control calcium signaling and contraction in the heart? *J. Mol. Cell. Cardiol.* **49,** 901–903 (2010).
3. Mozaffarian, D. *et al.* Heart Disease and Stroke Statistics—2016 Update. *Circulation* **133,** e38–e360 (2016).
4. White, H. D. & Chew, D. P. Acute myocardial infarction. *Lancet* **372,** 570–584 (2008).
5. Neri, M., Riezzo, I., Pascale, N., Pomara, C. & Turillazzi, E. Ischemia/Reperfusion Injury following Acute Myocardial Infarction: A Critical Issue for Clinicians and Forensic Pathologists. *Mediators Inflamm.* **2017,** 1–14 (2017).
6. Braunwald, E. & Kloner, R. A. Myocardial reperfusion: A double-edged sword? *J. Clin. Invest.* **76,** 1713–1719 (1985).
7. Bonnemeier, H. *et al.* Temporal repolarization inhomogeneity and reperfusion arrhythmias in patients undergoing successful primary percutaneous coronary intervention for acute ST-segment elevation myocardial infarction: Impact of admission troponin T. *Am. Heart J.* **145,** 484–492 (2003).
8. Fröhlich, G. M., Meier, P., White, S. K., Yellon, D. M. & Hausenloy, D. J. Myocardial reperfusion injury: looking beyond primary PCI. *Eur. Heart J.* **34,** 1714–1722 (2013).
9. Noble, D. & Herchuelz, A. Role of Na/Ca exchange and the plasma membrane Ca(2+)-ATPase in cell function. Conference on Na/Ca Exchange. *EMBO Rep.* **8,** 228–232 (2007).
10. Tanaka, H. *et al.* Involvement of the Na+/Ca2+ exchanger in ouabain-induced inotropy and arrhythmogenesis in guinea-pig myocardium as revealed by SEA0400. *J. Pharmacol. Sci.* **103,** 241–246 (2007).
11. Chen, S. & Li, S. The Na+/Ca^{2}+ exchanger in cardiac ischemia/reperfusion injury. *Med. Sci. Monit.* **18,** RA161-5 (2012).
12. Wanichawan, P. *et al.* Full-length cardiac Na+/Ca2+ exchanger 1 protein is not phosphorylated by protein kinase A. *Am. J. Physiol. Cell Physiol.* **300,** C989-97 (2011).
13. Allen, D. Role of the cardiac Na+/H+ exchanger during ischemia and reperfusion. *Cardiovasc. Res.* **57,** 934–941 (2003).
14. Vandenberg, J. I., Metcalfe, J. C. & Grace, A. A. Mechanisms of pHi recovery after global ischemia in the perfused heart. *Circ Res* **72,** 993–1003 (1993).
15. Wakabayashi, S., Shigekawa, M. & Pouyssegur, J. Molecular physiology of vertebrate Na+/H+ exchangers. *Physiol. Rev.* **77,** 51–74 (1997).
16. Marban, E., Yamagishi, T. & Tomaselli, G. F. Structure and function of voltage-gated sodium channels. *J. Physiol.* 647–57 (1998). doi:10.1111/j.1469-7793.1998.647bp.x
17. Chadda, K. R., Jeevaratnam, K., Lei, M. & Huang, C. L.-H. Sodium channel biophysics, late sodium current and genetic arrhythmic syndromes. *Pflugers Arch.* **469,** 629–641 (2017).
18. Maltsev, V. A. *et al.* Novel, Ultraslow Inactivating Sodium Current in Human Ventricular Cardiomyocytes. *Circulation* **98,** 2545–2552 (1998).
19. Ronchi, C. *et al.* Late sodium current and intracellular ionic homeostasis in acute ischemia. *Basic Res. Cardiol.* **112,** 1–12 (2017).
20. Ma, J. *et al.* Calmodulin kinase II and protein kinase C mediate the effect of increased intracellular calcium to augment late sodium current in rabbit ventricular myocytes. *Am. J.*

Physiol. Physiol. **302,** C1141–C1151 (2012).
21. Tang, Q. *et al.* Persistent sodium current and Na+/H+ exchange contributes to the augmentation of the reverse Na+/Ca2+ exchange during hypoxia or acute ischemia in ventricular myocytes. *Pflügers Arch. - Eur. J. Physiol.* **463,** 513–522 (2012).
22. Ma, J. *et al.* Ranolazine attenuates hypoxia- and hydrogen peroxide-induced increases in sodium channel late openings in ventricular myocytes. *J. Cardiovasc. Pharmacol.* **64,** 60–68 (2014).
23. Song, Y., Shryock, J. C., Wagner, S., Maier, L. S. & Belardinelli, L. Blocking Late Sodium Current Reduces Hydrogen Peroxide-Induced Arrhythmogenic Activity and Contractile Dysfunction. *J. Pharmacol. Exp. Ther.* **318,** 214 LP-222 (2006).
24. Joyce, S. How Much Money Do Sportswriters Make a Year? *Chron* (2017). at <http://work.chron.com/much-money-sportswriters-make-year-24715.html>
25. Undrovinas, A. I., Fleidervish, I. A. & Makielski, J. C. Inward sodium current at resting potentials in single cardiac myocytes induced by the ischemic metabolite lysophosphatidylcholine. *Circ. Res.* **71,** 1231 LP-1241 (1992).
26. Haufe, V. *et al.* Expression pattern of neuronal and skeletal muscle voltage-gated Na(+) channels in the developing mouse heart. *J. Physiol.* **564,** 683–696 (2005).
27. Hund, T. J. *et al.* A β IV -spectrin / CaMKII signaling complex is essential for membrane excitability in mice. **120,** (2010).
28. Mohler, P. J. & Hund, T. J. Role of CaMKII in cardiovascular health, disease, and arrhythmia. *Hear. Rhythm* **8,** 142–144 (2011).
29. Hoch, B., Meyer, R., Hetzer, R., Krause, E. & Karczewski, P. Identification and Expression of -Isoforms of the. *Processing* (1999).
30. Said, M. *et al.* Calcium-calmodulin dependent protein kinase II (CaMKII): A main signal responsible for early reperfusion arrhythmias. *J. Mol. Cell. Cardiol.* **51,** 936–944 (2011).
31. Feng, Y., Cheng, J., Wei, B. & Wang, Y. CaMKII inhibition reduces isoproterenol-induced ischemia and arrhythmias in hypertrophic mice. *Oncotarget* **8,** 17504–17509 (2017).
32. Hund, T. J. *et al.* A β IV -spectrin / CaMKII signaling complex is essential for membrane excitability in mice. *J. Clin. Invest.* **120,** 3508–3519 (2010).
33. Onal, B., Gratz, D. & Hund, T. J. Ca 2+ /calmodulin-dependent kinase II-dependent regulation of atrial myocyte late Na + current, Ca 2+ cycling, and excitability: a mathematical modeling study. *Am. J. Physiol. Circ. Physiol.* **313,** H1227–H1239 (2017).
34. Rook, M. B., Evers, M. M., Vos, M. A. & Bierhuizen, M. F. A. Biology of cardiac sodium channel Nav1.5 expression. *Cardiovasc. Res.* **93,** 12–23 (2012).
35. Remme, C. A. *et al.* The cardiac sodium channel displays differential distribution in the conduction system and transmural heterogeneity in the murine ventricular myocardium. *Basic Res. Cardiol.* **104,** 511–522 (2009).
36. Glynn, P. *et al.* Voltage-Gated Sodium Channel Phosphorylation at Ser571 Regulates Late Current, Arrhythmia, and Cardiac Function in vivo. *Circulation* **132,** 567–577 (2015).
37. Signore, S. *et al.* Late Na + current and protracted electrical recovery are critical determinants of the aging myopathy. *Nat. Commun.* **6,** (2015).
38. Hund, T. J. *et al.* A βIV-spectrin/CaMKII signaling complex is essential for membrane excitability in mice. *J. Clin. Invest.* **120,** 3508–3519 (2010).
39. Swift, L. M. *et al.* Properties of blebbistatin for cardiac optical mapping and other imaging applications. *Pflügers Arch. - Eur. J. Physiol.* **464,** 503–512 (2012).

40. Arora, R., Das, M. K., Zipes, D. P. & Wu, J. Optical mapping of cardiac arrhythmias. *Indian Pacing Electrophysiol J* **3,** 187–196 (2003).
41. Wang, L. *et al.* Optical mapping of sarcoplasmic reticulum Ca2+ in the intact heart: Ryanodine receptor refractoriness during alternans and fibrillation. *Circ. Res.* **114,** 1410–1421 (2014).
42. Attin, M. & Clusin, W. T. Basic Concepts of Optical Mapping Techniques in Cardiac Electrophysiology. *Biol Res Nurs* **11,** 195–207 (2009).
43. Koval, O. M. *et al.* Ca2+/calmodulin-dependent protein kinase ii-based regulation of voltage-gated na+ channel in cardiac disease. *Circulation* **126,** 2084–2094 (2012).
44. Hund, T. J. *et al.* βIV-Spectrin regulates TREK-1 membrane targeting in the heart. *Cardiovasc. Res.* **102,** 166–175 (2014).
45. Koval, O. M. *et al.* CaMKII-Based Regulation of Voltage-Gated Na(+) Channel in Cardiac Disease. *Circulation* **126,** 10.1161/CIRCULATIONAHA.112.105320 (2012).
46. Mitsuyama, H. *et al.* Ca2+/calmodulin-dependent protein kinase II increases the susceptibility to the arrhythmogenic action potential alternans in spontaneously hypertensive rats. *Am. J. Physiol. Heart Circ. Physiol.* **307,** H199-206 (2014).
47. Said, M. *et al.* Increased intracellular Ca(2+) and SR Ca(2+) load contribute to arrhythmias after acidosis in rat heart. Role of Ca(2+)/calmodulin-dependent protein kinase II. *Am. J. Physiol. - Hear. Circ. Physiol.* **295,** H1669–H1683 (2008).
48. Quinn, T. A. *et al.* Electrotonic coupling of excitable and nonexcitable cells in the heart revealed by optogenetics. *Proc. Natl. Acad. Sci.* **113,** 14852–14857 (2016).
49. Furukawa, T., Kimura, S., Furukawa, N., Bassett, A. L. & Myerburg, R. J. Role of cardiac ATP-regulated potassium channels in differential responses of endocardial and epicardial cells to ischemia. *Circ. Res.* **68,** 1693–1702 (1991).
50. Chou, C. C., Chang, P. C., Wei, Y. C. & Lee, K. Y. Optical Mapping Approaches on Muscleblind-Like Compound Knockout Mice for Understanding Mechanistic Insights Into Ventricular Arrhythmias in Myotonic Dystrophy. *J. Am. Heart Assoc.* **6,** 1–13 (2017).
51. O'Gara, P. T. *et al.* 2013 ACCF/AHA Guideline for the Management of ST-Elevation Myocardial Infarction: A Report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *Circulation* **127,** e362–e425 (2013).
52. Francis Stuart, S. D., De Jesus, N. M., Lindsey, M. L. & Ripplinger, C. M. The crossroads of inflammation, fibrosis, and arrhythmia following myocardial infarction. *J. Mol. Cell. Cardiol.* **91,** 114–122 (2016).
53. Mozaffarian, D. *et al. AHA Statistical Update Heart Disease and Stroke Statistics — 2016 Update A Report From the American Heart Association WRITING GROUP MEMBERS*. (2016). doi:10.1161/CIR.0000000000000350
54. Turer, A. T. & Hill, J. A. Pathogenesis of Myocardial Ischemia-Reperfusion Injury and Rationale for Therapy. *Am. J. Cardiol.* **106,** 360–368 (2010).
55. Baines, C. P. How and When Do Myocytes Die During Ischemia and Reperfusion: The Late Phase. *J. Cardiovasc. Pharmacol. Ther.* **16,** 239–243 (2011).
56. Kong, L.-H., Gu, X.-M., Wu, F., Jin, Z.-X. & Zhou, J.-J. CaMKII inhibition mitigates ischemia/reperfusion-elicited calpain activation and the damage to membrane skeleton proteins in isolated rat hearts. *Biochem. Biophys. Res. Commun.* **491,** 687–692 (2017).
57. Anderson, M. E., Brown, J. H. & Bers, D. M. CaMKII in myocardial hypertrophy and heart failure. *J Mol Cell Cardiol* **51,** 468–473 (2012).

58. Klabunde, R. E. Sodium-Channel Blockers (Class I Antiarrhythmics). (2011). at <http://www.cvpharmacology.com/antiarrhy/sodium-blockers.htm>
59. Nerbonne, J. M. Studying cardiac arrhythmias in the mouse - A reasonable model for probing mechanisms? *Trends in Cardiovascular Medicine* **14,** 83–93 (2004).
60. Kamiyama, T., Tanonaka, K., Harada, H., Nakai, K. & Takeo, S. Mexiletine and lidocaine reduce post-ischemic functional and biochemical dysfunction of perfused hearts. *Eur. J. Pharmacol.* **272,** 151–158 (1995).
61. NZ, K. *et al.* Mexiletine: Long-term efficacy and side effects in patients with chronic drug-resistant potentially lethal ventricular arrhythmias. *Arch. Intern. Med.* **150,** 381–384 (1990).
62. Anderson, M. E. Multiple downstream proarrhythmic targets for calmodulin kinase II: Moving beyond an ion channel-centric focus. *Cardiovasc. Res.* **73,** 657–666 (2007).
63. Erickson, J. R. & Anderson, M. E. CaMKII and Its Role in Cardiac Arrhythmia. *J. Cardiovasc. Electrophysiol.* **19,** 1332–1336 (2008).
64. van Oort, R. J. *et al.* Ryanodine Receptor Phosphorylation by Calcium/Calmodulin-Dependent Protein Kinase II Promotes Life-Threatening Ventricular Arrhythmias in Mice With Heart Failure. *Circulation* **122,** 2669–2679 (2010).
65. Wu, Y. *et al.* Calmodulin kinase II and arrhythmias in a mouse model of cardiac hypertrophy. *Circulation* **106,** 1288–1293 (2002).
66. Vincent, K. P., McCulloch, A. D. & Edwards, A. G. Toward a hierarchy of mechanisms in CaMKII-mediated arrhythmia. *Frontiers in Pharmacology* **5,** 110 (2014).
67. Bell, J. R., Vila-Petroff, M. & Delbridge, L. M. D. CaMKII-dependent responses to ischemia and reperfusion challenges in the heart. *Front. Pharmacol.* **5 MAY,** 1–7 (2014).
68. Di Carlo, M. N. *et al.* CaMKII-dependent phosphorylation of cardiac ryanodine receptors regulates cell death in cardiac ischemia/reperfusion injury. *J. Mol. Cell. Cardiol.* **74,** 274–283 (2014).
69. Imahashi, K. *et al.* Intracellular sodium accumulation during ischemia as the substrate for reperfusion injury. *Circ. Res.* **84,** 1401–1406 (1999).
70. Jung, I. *et al.* Cardioprotective effects of the novel Na+/H+ exchanger-1 inhibitor KR-32560 in a perfused rat heart model of global ischemia and reperfusion: Involvement of the Akt-GSK-3β cell survival pathway and antioxidant enzyme. *Arch. Pharm. Res.* **33,** 1241–1251 (2010).
71. Le Grand, B. *et al.* Sodium Late Current Blockers in Ischemia Reperfusion: Is the Bullet Magic? *J. Med. Chem.* **51,** 3856–3866 (2008).
72. Undrovinas, A. & Maltsev, V. a. Late sodium current is a new therapeutic target to improve contractility and rhythm in failing heart. *Cardiovasc. Hematol. Agents Med. Chem.* (2008). doi:10.2174/187152508785909447
73. Veerman, C. C. *et al.* The Brugada Syndrome Susceptibility Gene HEY2 Modulates Cardiac Transmural Ion Channel Patterning and Electrical Heterogeneity. *Circ. Res.* **121,** 537–548 (2017).
74. Imahashi, K. Cardiac-Specific Ablation of the Na+-Ca2+ Exchanger Confers Protection Against Ischemia/Reperfusion Injury. *Circ. Res.* **97,** 916–921 (2005).
75. MacDonald, A. C. & Howlett, S. E. Differential effects of the sodium calcium exchange inhibitor, KB-R7943, on ischemia and reperfusion injury in isolated guinea pig ventricular myocytes. *Eur. J. Pharmacol.* **580,** 214–223 (2008).
76. Makielski, J. C. 'Late sodium current: a mechanism for angina, heart failure, and

arrhythmia'. *J. Cardiovasc. Pharmacol.* **54,** 279–286 (2009).
77. Hale, S. L., Leeka, J. A. & Kloner, R. A. Improved left ventricular function and reduced necrosis after myocardial ischemia/reperfusion in rabbits treated with ranolazine, an inhibitor of the late sodium channel. *J. Pharmacol. Exp. Ther.* **318,** 418–23 (2006).
78. Frommeyer, G., Milberg, P., Maier, L. S. & Eckardt, L. Late sodium current inhibition: the most promising antiarrhythmic principle in the near future? *Curr. Med. Chem.* **21,** 1271–80 (2014).
79. Tilz, R. R., Lin, T., Makimoto, H. & Ouyang, F. Successful epicardial ablation of electrical storms due to recurrent ventricular fibrillation triggered by premature ventricular contractions. *Hear. Rhythm* **11,** 146–149 (2014).
80. Investigators, T. C. A. S. T. (CAST). Preliminary report: effect of encainide and flecainide on mortality in a randomized trial of arrhythmia suppression after myocardial infarction. *N. Engl. J. Med.* **321,** 406–412 (1989).
81. Nattel, S. *et al.* New directions in cardiac arrhythmia management: present challenges and future solutions. *Can. J. Cardiol.* **30,** S420-30 (2014).
82. Aldakkak, M., Camara, A. K. S., Heisner, J. S., Yang, M. & Stowe, D. F. Ranolazine reduces Ca2+ overload and oxidative stress and improves mitochondrial integrity to protect against ischemia reperfusion injury in isolated hearts. *Pharmacol. Res.* **64,** 381–392 (2011).
83. Calderó N-Sá Nchez, E. M. *et al.* Cardioprotective Effect of Ranolazine in the Process of Ischemia-reperfusion in Adult Rat Cardiomyocytes. *Rev Esp Cardiol* **69,** 45–53 (2016).
84. Dhalla, A. K. *et al.* Ranolazine, an antianginal agent, markedly reduces ventricular arrhythmias induced by ischemia and ischemia-reperfusion. *AJP Hear. Circ. Physiol.* **297,** H1923–H1929 (2009).
85. Koenig, X. *et al.* Enhanced currents through L-type calcium channels in cardiomyocytes disturb the electrophysiology of the dystrophic heart. *Am. J. Physiol. Heart Circ. Physiol.* **306,** H564–H573 (2014).
86. Gandhi, A. *et al.* The effect of SN-6, a novel sodium-calcium exchange inhibitor, on contractility and calcium handling in isolated failing rat ventricular myocytes. *Cardiovasc. Ther.* **31,** 115–124 (2013).
87. Niu, C. F. *et al.* Characterization of SN-6, a novel Na+/Ca2+exchange inhibitor in guinea pig cardiac ventricular myocytes. *Eur. J. Pharmacol.* **573,** 161–169 (2007).
88. Gao, E. *et al.* A Novel and Efficient Model of Coronary Artery Ligation and Myocardial Infarction in the Mouse. *Circ. Res.* **107,** 1445–1453 (2010).

8. Figure Legends

Figure 1: **(A)** Proposed hypothesis linking CaMKII-dependent phosphorylation of Nav1.5 and arrhythmia susceptibility; **(B)** Experimental protocol to study arrhythmia susceptibility in isolated, Langendorff-perfused mouse hearts.

Figure 2: (A) Representative immunoblots and **(B)** densitometric measurements for phosphorylated CaMKII and phosphorylated $Na_V1.5$ (expressed as ratio to total CaMKII and $Na_v1.5$, respectively) in wild- type (WT) mice at control, post ischemia (Isc) and post reperfusion (I/R) time points heart tissues. *P<0.05 vs. baseline; #P<0.05 vs. reperfusion; (n=3 in each group).

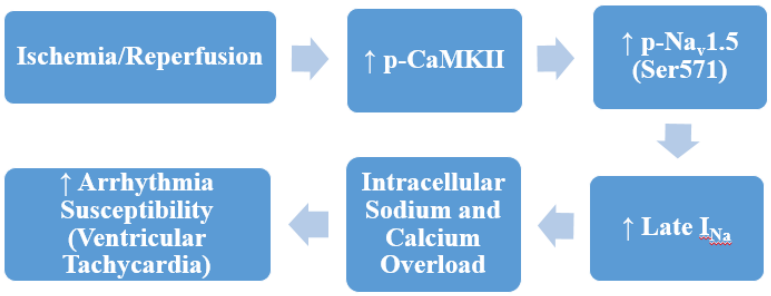
Figure 3: (A) Optically recorded action potentials and **(B)** summary data for AP duration at 80% repolarization (APD) in WT, S571E and S571A hearts at baseline and during I/R protocol. Pacing cycle length (PCL) of 150 ms was used for all measurements. * $p < 0.05$ vs WT, # $p < 0.05$ vs SA. n=5 independent preparations for all groups. Scale bar = 50ms.

Figure 4: (A) Summary data and **(B)** representative Aps during S1S2 protocol to induce arrhythmia following I/R for wild- type (WT), S571 mutant (SE), and S571 mutant (SA) hearts. Pacing cycle length (PCL) of 150 ms was used for all measurements. Red arrow denotes initiation of S2 protocol. * $p < 0.05$ vs WT, # $p < 0.05$ vs SA. n=5 independent preparations for all groups.

Figure 5: Shortening of action potential duration (APD) in wild-type (WT) mice treated with Mexiletine. A, Representative action potential overlay for WT and WT drug hearts is shown at Baseline, Post Ischemia, and Post Reperfusion. Pacing cycle length (PCL) of 150 ms was used for all measurements. * $p < 0.05$ vs WT, # $p < 0.05$ vs SA. n=5 independent preparations for all groups. Scale bar = 50ms. B, Percent change of the APD measured for APD_{80} from Baseline to Post Ischemia at 15 minutes, and Baseline to Post Reperfusion at 5 minutes for wild- type (WT), and wild-type with drug hearts.

Figure 6: Decreased arrhythmia susceptibility in wild-type (WT) mice treated with Mexiletine.

9. Figures



Protocol	Baseline Recordings	Ischemia (Stop Flow)	Reperfusion and S1-S2 (Restart Flow)
Duration (minutes)	2	15	15
Pacing Cycle Length (ms)	150	150	150, 150-X

Figure 1

Figure 1

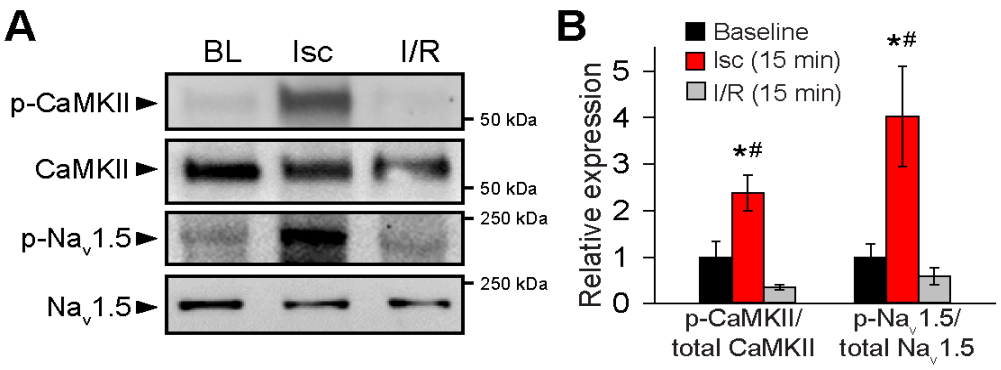


Figure 2

Figure 2

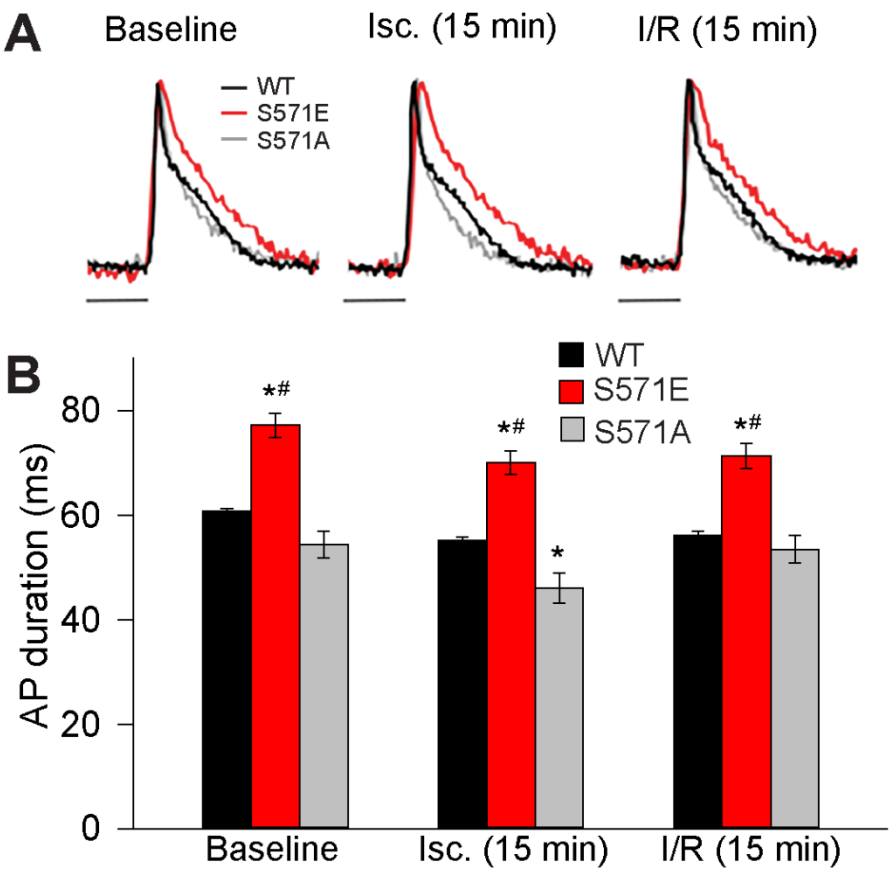


Figure 3

Figure 3

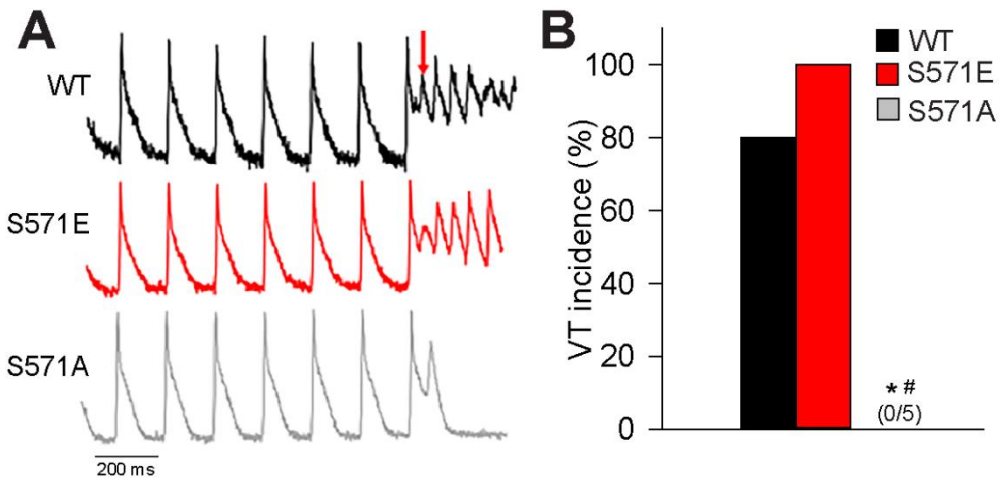


Figure 4

Figure 4

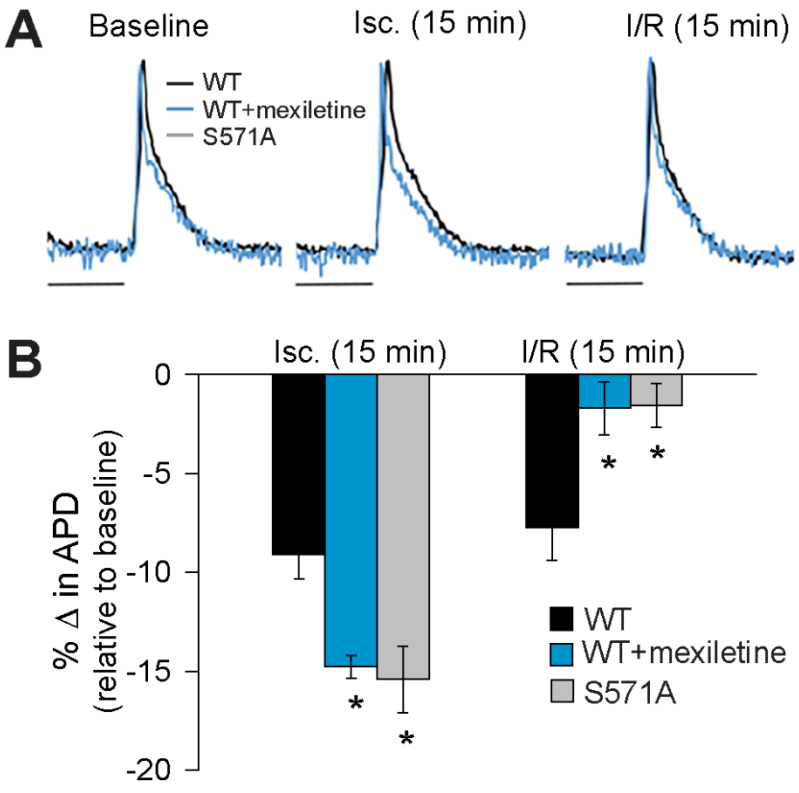


Figure 5

Figure 5

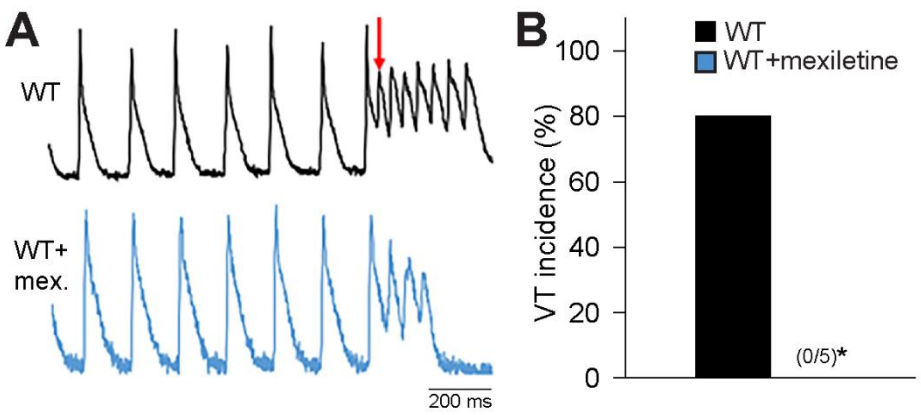


Figure 6

Figure 6